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Efficacy of suicide gene therapy in hypoxic rat 9L glioma cells

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Abstract

Viral vector mediated suicide gene therapy (SGT) involving thymidine kinase (TK) or cytosine deaminase (CD) have considerable promise in the treatment of malignant brain tumors. An unresolved issue is to what extent tumor hypoxia influences the outcome of SGT since brain tumors characterized by regions of hypoxia have potentially reduced cellular metabolism and SGT's cytotoxicity is manifest through cellular metabolism. We studied in vitro and in vivo, the effect of hypoxia on the cytotoxicity of SGT in rat 9L glioma cells. Neither acute nor chronic hypoxia affected the cell killing of SGT by TK or CD. In vivo confirmation that SGT efficacy was not adversely affected by tumor hypoxia using the hypoxic cell marker pimonidazole was shown by the absence of a change in tumor hypoxia by SGT. These studies support the use of SGT utilizing either TK or CD gene strategies even when tumors are characterized by a hypoxic microenvironment.

Keywords

Cytosine deaminase; Hypoxia; Pimonidazole; Suicide gene therapy; Thymidine kinase

Introduction

Suicide gene therapy is currently being tested in clinical trials for a variety of human cancers, including cancer of the prostate and malignant brain tumors [1, 2]. This approach is based on the premise that the cells expressing the suicide genes will be rendered sensitive to prodrugs, thereby, increasing the therapeutic index. Two suicide genes which are being tested are the *E. coli* cytosine deaminase (CD) and HSV-1 thymidine kinase (TK) genes, which confer sensitivity to 5-fluorocytosine (5-FC) and ganciclovir (GCV), respectively.

In our earlier studies, we have shown that the combined suicide gene therapy and radiation therapy were effective means to improve the efficacy of radiation therapy [3, 4]. Using both human glioma cells in culture and 9L gliosarcoma in vivo, significant potentiation of tumor radiosensitivity was achieved. However, all the foregoing pre-clinical studies were carried out with tumor cells under well-oxygenated conditions.

Since most high grade malignant gliomas harbor varying degrees of hypoxic and anoxic tumors cells [5, 6], which are highly radioresistant, the issue of relative effectiveness of suicide gene therapy against hypoxic tumor cells would become clinically relevant. Since the activity of enzymes used in SGT depends on cellular metabolism and pH, both of which are directly affected by oxygen status of the cell, the importance of tumor hypoxia on suicide gene therapy efficacy assumes significance. Although others have addressed the

issue of cell killing efficiency under hypoxic conditions following suicide gene therapy [7], no studies have been carried out with malignant glioma cells in in vivo setting.

The present study was initiated to determine if hypoxia, either acute or chronic exposure affects the efficacy of SGT in 9L glioma cells. In vivo studies using hypoxic cell marker pimonidazole was carried out to determine the effect of hypoxia on SGT cytotoxicity.

Materials/methods

Effect of hypoxia in vivo

Cell line—9L cells were retrovirally transduced with the CD/TK fusion gene as described previously [8], to stably express two suicide genes, E. Coli cytosine deaminase, CD and Herpes Simplex Virus Type-1 thymidine kinase, TK, each capable of converting prodrugs, 5-fluorocytosine (5-FC) and antiviral drugs such as ganciclovir, into toxic metabolites, 5-fluorouracil (5-FU) and ganciclovir monophosphate, respectively.

Stably transduced 9L cells were cultured in Dulbecco's Eagles' medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin and streptomycin, and 800 µg/ml of G418 for selection (Gibco, Rockville, MD) and 400 µg/ml G418 for continued selection pressure of the transduced genotype as described previously [9]. Cells were maintained as a monolayer culture at 95% ambient air, 5% CO₂ and 37°C.

Hypoxia condition—Experimental conditions were designed to achieve radiobiological hypoxia under acute, 1 h, or chronic, 24–48 h of exposure. Ten-thousand cells were placed on T-15 flasks (Bellco Glass Inc., Vineland, NJ), in the presence or absence of prodrugs, exposed to 95% N₂ + 5% CO₂ for 1 h to achieve acute hypoxia, and subsequently returned to oxygenated culture conditions for the duration of the study. For chronic hypoxic experiments, the cultured flasks were purged with 95% N₂ + 5% CO₂ for 1 h prior to sealing for the remaining 23 (or 47) h. Hypoxic cells, either acutely or chronically exposed were incubated with the required concentration of 5-FC or GCV during the hypoxic exposure and in the time following.

Endpoint—Cytotoxicity was assessed by clonogenic assay to determine the cell killing efficiency of two suicide genes under hypoxic conditions. Experiments were carried out in triplicate cell culture plates and repeated a minimum of three times. Error bars on cell survival curves represent standard deviation of the means.

Tumor hypoxia in vivo

The tumor hypoxia was assessed in vivo by estimating the percent tumor area stained positively with pimonidazole, a well established marker that gives a reliable estimate of radio biologically relevant hypoxia [10, 11]. The quantification of tumor hypoxic fraction based on pimonidazole staining has been used before [12–15].

24 male athymic mice (CD1nu/nu) aged 5–6 weeks were obtained from Charles River laboratory. The animals were inoculated subcutaneously in the right hind limb with stably transduced 9L glioma cells carrying double suicide genes (E. coli CD and HSV-1 TK). 12 mice were treated with the prodrugs and 12 were untreated controls. All studies were conducted in an AAALAC approved facility according to the Institutional animal care and use committee guidelines.

Prodrugs treatment and pimonidazole administration

Animals in the treatment group were treated with 500 mg/kg of 5-FC and 30 mg/kg of GCV intra peritoneally once a day for 4 days at which time the tumors were harvested as previously described [3]. Tumor response to SGT was noted by measuring the tumor size every day during the treatment period. Ninety minutes before tumor harvest, animals were treated with 75 mg/kg Pimonidazole (Chemicon Int, Temecula, CA) intraperitoneally.

Specimen preparation—After animals were euthanized, tumors were removed and flash frozen in 2-methyl butane at -40°C . Tumor specimens were transferred to a cryostat and cut as cross-sectional slices of approximately 10 μm thick.

Pimonidazole staining—Pimonidazole uptake was detected using the Hypoxyprobe-1 Kit (Chemicon Int, Temecula, CA). Five sections from each tumor, separated at regular intervals spaced a millimeter apart, were stained with pimonidazole. Slides were initially fixed with 10% formalin for 10 min and washed in distilled water. Endogenous peroxidase was blocked using 3% H_2O_2 for 5 min and washed. Protein block (DAKO Corp, CA) was added and left for 5 min to inhibit non-specific staining. Anti-pimonidazole primary antibody (Hypoxyprobe-1Mab1) diluted 1/50 in PBS was added for two hours at room temperature. Sections were then washed thoroughly and incubated with biotin-conjugated F(ab)2 secondary antibody reagent (Chemicon Int, Temecula, CA) for 10 min. Sections were washed before the addition of diaminobenzidine substrate DAB (DAKO Corp, CA). This was left for 5 min and washed in distilled water. Sections were then placed in hematoxylin for 30 s and then washed well in distilled water. Sections were dehydrated in an ascending ethanol series, immersed in xylene, and coverslipped with Permount (Fisher Scientific, PA). Cells with bound pimonidazole stained brown. A negative control was used during each staining run and consisted of the substitution of the anti pimonidazole antibody for an isotypic control antibody.

To further facilitate accurate and quantitative detection of hypoxic regions we employed a primary mouse antibody directed against pimonidazole protein and a secondary mouse antibody conjugated to fluorescein isothiocyanate-FITC (Vector labs, CA).

Data analysis

For quantitative image analysis of immunostaining with pimonidazole, multiple images (between 100 and 150 depending on the cross-sectional tumor size) from each section were obtained at 10 $\times$ magnification (Olympus, PA). Composite image of the entire tumor tissue was prepared by tiling the individual images. The fractional area stained with pimonidazole was estimated as described previously [14]. Briefly, thresholding was performed to differentiate tissues from background and pimonidazole stained tissue from the rest of the image. The threshold intensities depended on the intensity of the staining. The hypoxic fraction was calculated by dividing the stained tissue area by the total tissue area after correcting for necrosis, acellularity and tissue artifacts.

Results

Effect of hypoxia

Radiobiological hypoxia was confirmed using the exponential part of clonogenic cell survival curves with or without oxygen to quantify the increased resistance of cells to radiation exposure under the experimental hypoxia conditions described above. Specifically, the radiation dose necessary to achieve the same percentage of surviving cells following either the acute or the chronic hypoxia exposure compared to the radiation dose under

similar normal oxygen conditions was more than two times higher demonstrating that the cells were radiobiologically hypoxic (oxygen enhancement ratio: 2.7 ± 0.3).

Acute or chronic hypoxia had no effect on cell killing by either CD or TK approaches compared to that obtained under oxygenated conditions. The dose-dependent cell killing by CD was unaffected by either acute hypoxia (Fig. 1a) or chronic hypoxia for 48 h (Fig. 1b). Identical survival results were obtained when cells were exposed to chronic hypoxia for 24 h (data not shown). Similarly the dose-dependent cell killing by TK was unaffected by either acute hypoxia (Fig. 2a) or chronic hypoxia for 24 h (Fig. 2b).

In vivo effect of hypoxia

The mean tumor area that stained positively with pimonidazole before prodrug administration was 7.2% ($\pm 0.9\%$) (Fig. 3a) compared to 6.8% ($\pm 1.1\%$) in the drug treated group (Fig. 3b) indicating that the hypoxic fraction (defined as the ratio of hypoxic area to total tumor area) did not change significantly after SGT. Similar results were obtained after staining different tissue sections from the tumor with FITC conjugated secondary mouse antibody (Fig. 3c).

It is of note that tumor size decreased by approximately 1 mm in diameter per day during SGT suggesting tumor response to treatment. In comparison, untreated controls increased in tumor size by approximately 1 mm in diameter per day (Fig. 4).

Discussion

The data presented here indicate that the efficacy of CD and TK mediated suicide gene therapy is not reduced due to either acute or chronic hypoxia. Furthermore, in our in vivo studies we observed that the hypoxic fraction did not change significantly between SGT treated and untreated groups indicating equal SGT cytotoxicity regardless of oxygenation or hypoxia conditions. The results are important because they suggest that SGT may well be effective against cancer cells characterized by hypoxia.

A cytotoxic therapy that selectively acts on well oxygenated cells will increase the hypoxic fraction after therapy. Classical radiobiological studies have shown that radiation preferentially kills well oxygenated cells over hypoxic cells [16, 17]. As a result this differential toxicity, the fraction of hypoxic cells would increase after radiation [18]. This is true for most solid tumors that harbor variable degree of hypoxia. In the present study we tested the cytotoxic efficacy of SGT in presence of hypoxia by comparing the change in hypoxic fraction before and after prodrug administration. Under identical experimental conditions, if the prodrugs/suicide gene therapy combination did not work in the hypoxic regions but only in oxygenated regions, the tumor hypoxic fraction would increase. After drug treatment, there was a tumor response (Fig. 4) but no significant change in the hypoxic fraction (Fig. 3b) compared to untreated controls (Fig. 3a). This is possible when SGT is effective both in hypoxic and oxygenated regions of the tumor. If SGT was effective only in oxygenated regions, the hypoxic fraction would be higher in the treated group.

Gene therapy approaches designed to target hypoxic tumor regions either by using anaerobes incorporating suicide genes [19, 20] or hypoxia inducible promoters to up-regulate either CD or TK suicide gene expression [21–23] have been developed. But the question is whether the up regulation of CD or TK genes would enhance the SGT in regions of low oxygen tension. Recently there have been studies about the efficacy of CD/5-FC system under hypoxic conditions. A previous report has demonstrated that the cells expressing CD maintained under conditions of chronic hypoxia showed an increase in cell killing with increases in concentration of 5-FC [7]. The current study confirms and extends

the previous report since we used two suicide genes and showed no appreciable difference in cell killing between oxic and hypoxic conditions and demonstrated in vivo evidence that hypoxia does not affect SGT efficacy.

An issue worth considering is whether prodrugs are capable of reaching hypoxic tumor regions. Since we noted tumor response to SGT, the observed results suggest that the drug doses used in the in vivo studies were sufficient. Although prodrugs such as 5-FC and GCV do cross the blood brain barrier, high interstitial pressure characteristic of tumors represent a theoretical barrier [24]. In fact, our pre-clinical studies with orthotopic brain tumors indicate SGT to be effective against small tumors and when combined with radiotherapy showed efficacy in larger tumors [3].

The greatest clinical significance that SGT has is when combined with radiotherapy. A benefit of the CD/TK fusion suicide gene therapy approach is the enhanced cytotoxicity in the presence of ionizing radiation at significantly lower concentrations than needed for direct cytotoxicity. Early clinical results from our center demonstrates no dose limiting toxicity for SGT use in prostate cancer [25]. The results presented indicating that CD and TK mediated direct cytotoxicities are not adversely affected under hypoxic conditions may logically be extended to enhanced cytotoxicity of CD- and TK-mediated radio-sensitization under hypoxic conditions. Further studies are underway to confirm whether SGT using CD/TK approach offer increased radio-sensitization under conditions of tumor hypoxia.

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Fig. 1.

Cell survival is shown following suicide gene therapy with CD/5-FC over a range of concentrations under hypoxic (dotted line) or normal oxygen (solid line) conditions. Panel **a** corresponds to CD/5-FC toxicity under acute hypoxic condition. Panel **b** illustrates CD/5FC toxicity under conditions of chronic hypoxia for 48 h. Error bars are smaller than the symbol

Fig. 2.

Cell survival is shown following suicide gene therapy with TK/GCV. Cells stably expressing TK were exposed to prodrugs over a range of concentrations of GCV under hypoxic (dotted line) or normal oxygen (solid line) conditions. Panel **a** corresponds to TK/GCV toxicity under acute hypoxia. Panel **b** illustrates TK/GCV toxicity under condition of chronic hypoxia for 24 h. Error bars are smaller than the symbol

Fig. 3. Immunohistochemical staining using pimonidazole showing a representative tumor section before prodrugs treatment (**a**) compared to 4 days of prodrugs treatment (**b**). Pimonidazole stained region appear brown (shown by pointed arrow). (**c**) Pimonidazole conjugated with FITC (green)

Fig. 4. Effect of SGT in mouse tumor model. CD-1 nude mice bearing 9L-CD/TK tumors were treated with GCV (30 mg/kg) and 5-FC (500 mg/kg) for 4 days (solid line). Dotted line indicates untreated tumors during the same period. Error bars represent standard deviation of the mean